Reference: *Biol. Bull.* **193:** 14–19. (August, 1997)

# Chromosome Segregation in Fertilized Eggs From Triploid Pacific Oysters, *Crassostrea gigas* (Thunberg), Following Inhibition of Polar Body 1

HUAYONG QUE[1], XIMING GUO[2], FUSUI ZHANG[1], AND STANDISH K. ALLEN, JR.[2,*]

[1]*Institute of Oceanology, Chinese Academy of Sciences, Qingdao, Shandong 266071, P. R. China;*
[2]*Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Science, Rutgers University, Port Norris, New Jersey 08349*

**Abstract.** Chromosome segregation in fertilized eggs from triploid Pacific oysters, following inhibition of the first polar body (PB1), was studied with acetic orcein staining techniques. To block the release of PB1, fertilized eggs were treated with 0.5 mg/l of cytochalasin B (CB). Four types of segregation were observed, namely, "tripolar segregation" (54.5%), "united bipolar segregation" (12%), "separated bipolar segregation" (2.5%), and "incomplete united bipolar segregation" (4%). The remaining 23% could not be classified because of chromosome disorganization, but appeared to be variants of the above. It seemed clear that the predominant pattern that gave rise to tetraploids was united bipolar segregation, although certain separated bipolar segregations might also lead to the formation of tetraploids. The sequential events of meioses observed in CB-treated eggs are described. The asynchrony of meiotic events and possible mechanisms for the various types of chromosome segregation are discussed.

## Introduction

Viable tetraploids have been produced in the Pacific oyster, *Crassostrea gigas* by blocking the first polar body (PB1) in eggs from triploids fertilized with haploid sperm (Guo and Allen, 1994b). Previous studies of the effect of blocking PB1 on subsequent chromosome segregation in diploid eggs revealed a variety of segregation patterns during meiosis II, including "tripolar segregation," "united bipolar segregation," and "separated bipolar segregation" (Guo, 1991; Guo *et al.*, 1992). It was hypothesized that tetraploids were produced by united bipolar segregation. If this type of segregation occurred in a triploid egg, 30 chromosomes would remain in the egg, and with an additional 10 chromosomes contributed by a haploid sperm, tetraploidy would result (Guo, 1991). However, this hypothesis was not supported by cytological observations in triploid eggs themselves. Only recently have observations on triploid meioses been made (Guo and Allen, 1994a; Komaru and Wada, 1994). In Pacific oysters, Guo and Allen (1994a) found that the majority of fertilized eggs from triploid female × diploid male matings (TD crosses) went through two meiotic divisions and released two polar bodies, as did diploid eggs. Survivors consisted of 33% diploids, 5% triploids, and 10% tetraploids. In the tetraploid induction, when TD crosses were treated with cytochalasin B (CB)—so called TDCB crosses—aneuploids (23%), diploids (3%), triploids (3%), and mosaics (3%) were found in addition to tetraploids (67%) at 3 months postfertilization (PF) (Guo and Allen, 1994b). The differences in ploidy composition between TD and TDCB crosses demonstrated that blocking PB1 greatly changed the chromosome segregation in triploid eggs.

The objectives of this study were to reveal the pattern of meiotic segregation in fertilized eggs from triploids following the inhibition of PB1, document the probable cytological explanation for the production of tetraploids, and test the hypothesis that united bipolar segregation can occur in these zygotes.

Received 19 March 1997; accepted 13 May 1997.
* Corresponding author.

## Materials and Methods

Triploid Pacific oysters used in this study were 2 years old and produced by blocking the release of the second polar body (PB2) in the fertilized eggs of diploids (Allen *et al.*, 1989). Ploidy was confirmed in all individuals by flow cytometry prior to spawning. Gametes were obtained by strip-spawning. Eggs were passed through a 60-$\mu$m screen to remove the large tissue debris and rinsed on a 25-$\mu$m nytex screen. Sperm was passed through a 25-$\mu$m screen to remove tissue debris. All fertilization and incubation was conducted at 24° to 26°C using filtered (2 $\mu$m) seawater.

Two experimental groups were produced. In the first group, fertilized eggs were allowed to develop as controls, referred to as TD. In the second group, fertilized eggs were treated with 0.5 mg/l CB in 0.5% DMSO, referred to as TDCB. Treatment with CB began at the first indication of PB1 extrusion and lasted until about 50% of the developing eggs exhibited PB1 in the control. That is, the control was used to gauge the timing of the treatment group that was under the influence of CB. Two replicates were conducted using different pairs of parents.

To study chromosome segregation, samples of developing zygotes were taken every 5 min until 60 min PF and fixed with Carnoy's solution (1:3 glacial acetic acid and absolute methanol). Fixatives were changed twice following light centrifugation. Chromosomes were observed by acetic orcein stain (Guo *et al.*, 1992). Briefly, drops of fixed sample were spread on a slide, mixed with 2–3 drops of orcein stain (0.5% orcein in 60% acetic acid), and covered with a cover glass. After 5 min, the cover glass was pressed gently and sealed with Cytoseal mounting medium. Slides were examined with a Nikon Opiphot microscope. Photographs were taken with Kodak Technical Pan 2415 black-and-white film with speed set at 64 ASA.

## Results

Synapsis in eggs from triploid Pacific oyster was characterized by the presence of a mixture of trivalents, bivalents, and univalents, as well as multivalents greater than 3 (Fig. 1A). In other cases, nearly complete synapsis was indicated by the presence of about 10 multivalents, most appearing to be trivalents (Fig. 1B). Shortly after fertilization, the eggs resumed meiosis. Most eggs entered anaphase I around 20 min PF. Generally, replicated chromosomes (dyads) partitioned into two groups and moved toward opposite poles (Fig. 1C). We also observed that although most dyads underwent segregation at anaphase I, others remained isolated from the two segregating groups (Fig. 1D). By the time anaphase I was over, two groups of dyads had formed, each with variable numbers of chromosomes but averaging 15. The peripheral group became condensed during telophase I (around 30–35 min PF). Up to this stage, meiotic behavior in treated (TDCB) groups was essentially the same as in controls. Differences between the two groups appeared 35–45 min PF. In control (TD) groups, most eggs extruded the first polar body (PBI). In contrast, no polar bodies were observed in most eggs in TDCB groups. In TDCB, the peripheral group of chromosomes that was supposed to be released as PB1 moved back and combined with the inner group of chromosomes, ready for meiosis II. Four typical patterns of chromosome segregation could be classified during meiosis II.

### *Tripolar segregation*

The 30 dyads were delivered at random into three division planes in a tripolar configuration when the egg entered metaphase II (about 35–40 min PF) (Fig. 1E). During anaphase II (about 40–45 min PF), two chromatids of each chromosome detached and moved to the two adjacent poles independently (Fig. 1F). Each of the three poles received an average of 20 chromosomes from the two adjacent groups of dyads when telophase II was reached (about 45–50 min PF). However, the number of chromosomes was highly variable at each pole. After telophase II, all three groups of chromosomes became condensed. Although observations ceased before actual release of PB2, we suppose that the peripheral group of chromosomes was released as PB2 and that it is likely that PB2 sometimes contained two groups of chromosomes.

### *United bipolar segregation*

The two groups of dyads from meiosis I united completely and aligned on a single division plane in a bipolar configuration (Fig. 1G). The sister chromatids divided and segregated to the opposite poles independently (Fig. 1H). Telophase II ended with 30 chromosomes distributed at each pole. The peripheral group became packaged and compact (Fig. 1I) and supposedly was released as PB2 after telophase II.

### *Incomplete united bipolar segregation*

Dyads from meiosis I united completely and reorganized on a single division plane, except for a few dyads that were separated from the metaphase II division plane—"orphan chromosomes" (Fig. 1L). Subsequently, sister chromatids of dyads, which were grouped together on the division plane, separated from each other, moving to the poles independently. Concomitantly, the sister chromatids in orphan chromosomes also separated (Fig. 1M). There is no evidence that chromatids derived from orphans moved to either pole.

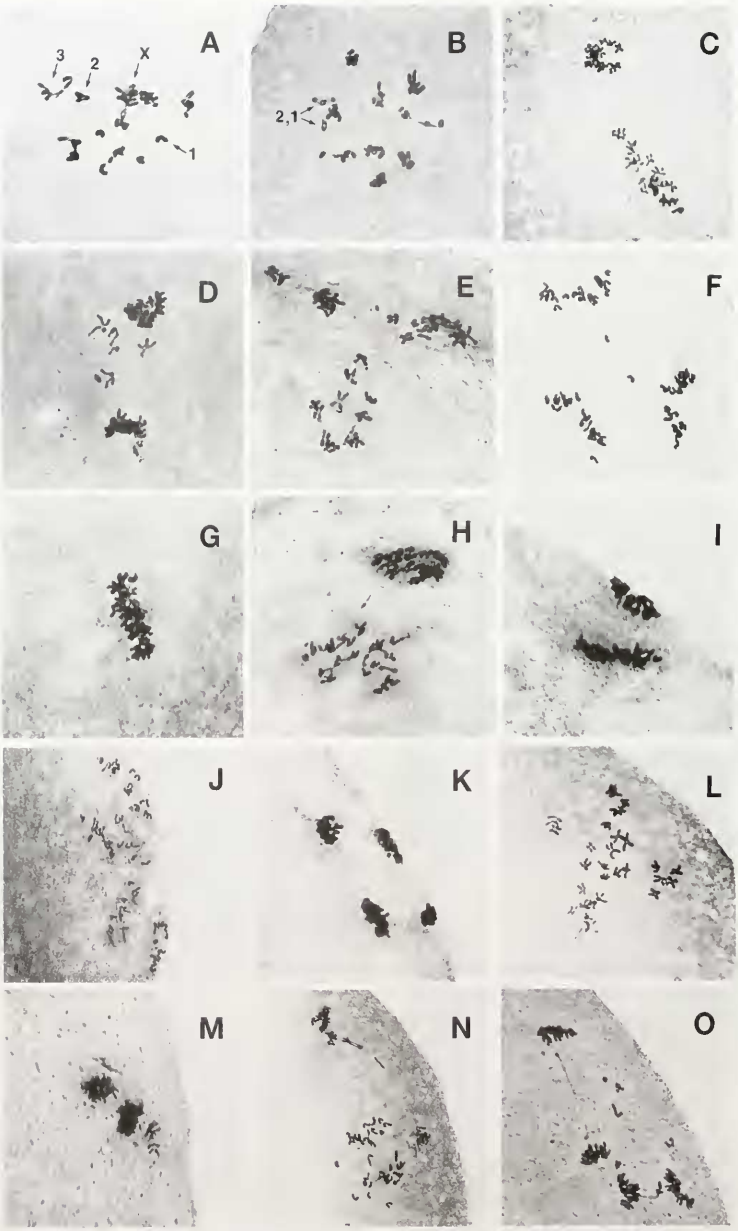


**Figure 1.** Chromosome segregation observed in fertilized eggs from triploid Pacific oysters, following inhibition of polar body 1. (A) mix of univalents (1), bivalents (2), trivalents (3), and multivalents (>3 = x) at synapsis. (B) Nearly complete synapsis, with 9 trivalents and a bi-, univalent combination (2,1). (C–D) Segregation at anaphase I. (E–F) Tripolar segregation. (G–I) United bipolar segregation. (J–K) Separated bipolar segregation. (L–M) Incomplete united bipolar segregation. (N–O) Intermediate segregation.

Rather, they remained in the area of the former metaphase II division plane.

*Separated bipolar segregation*

The two groups of dyads from meiosis I, rather than overlapping or uniting, entered meiosis II separately. Each group formed a bipolar configuration and then proceeded through chromosome migration (Fig. 1J). At the end of anaphase II, four groups of chromosomes were formed (Fig. 1K).

*Unclassified segregation*

In addition to the above four chromosome segregations, there were many intermediate segregations that could not be classified exactly. For example, we observed that one group of dyads from meiosis I went through meiosis II in a manner of bipolar segregation while the other group of dyads remained in its original state (Fig. 1N). We also found that in meiosis II, some chromosomes were left behind while others had already reached the corresponding poles and had become condensed. These aberrant and lagging chromosome movements occurred in what appeared to be either tripolar or separated bipolar configurations (Fig. 1O).

*Pattern frequencies*

The frequencies of the various segregation patterns were determined at telophase II by the number of maternal chromosome groups present (incomplete united bipolar segregation was identified by the presence of two groups of maternal chromosomes and some isolated chromosomes) (Table I). The majority of the treated eggs (54.5%) went through tripolar segregation.

**Table I**

*Percentage of chromosome segregation patterns observed in fertilized eggs of triploid* Crassostrea gigas *following inhibition of PB1, in two replicates*

| Replicate ($n$)* | Chromosome segregation patterns (%) | | | | |
|---|---|---|---|---|---|
| | Tripolar | United bipolar | Incomplete united bipolar | Separated bipolar | Unclassified patterns |
| 1 (100) | 56 | 13 | 4 | 2 | 25 |
| 2 (90) | 53 | 11 | 4 | 3 | 29 |
| mean | 54.5 | 12.0 | 4.0 | 2.5 | 27.0 |

* Number of observations.

## Discussion

Completeness of synapsis in triploid Pacific oyster varied considerably, as was also found in a previous study (Guo and Allen, 1994a). Our observations demonstrated that the general configurations of synapsis that Darlington (1965) described in triploid organisms also exist in triploid Pacific oysters. According to Darlington (1965), trivalents, bivalents, and univalents can be found in the same nucleus of triploid organisms. Such modalities of chromosome pairing were also found in humans (Gosden *et al.*, 1976; Luciani *et al.*, 1978). Other studies in triploid *Allium sphaerocephalon* revealed that only two of each set of three homologous chromosomes participated in synapsis, the other remained unsynapsed (Loidl and Jones, 1986). This might be an exception to the rule in triploid organisms. Variation in the degree of synapsis in eggs from triploids might influence subsequent meiotic events following inhibition of PB1, though it was difficult to exactly correlate the completeness of synapsis with subsequent types of chromosome segregation. Chromosome segregation in control groups seemed to be unaffected by completeness of synapsis: most of the eggs completed two meioses in a manner similar to that seen among diploid eggs—the extra set of chromosomes segregating randomly (Longo *et al.*, 1993; Guo and Allen, 1994a). It was also found that meiotic divisions in eggs from triploid Japanese pearl oyster (*Pinctada fucata martensii*) were virtually identical to those in the diploid control (Komaru and Wada, 1994).

Our observations also suggest that the meiotic events in CB-treated triploid eggs are remarkably asynchronous. Asynchrony was observed at three levels. First, there was variation in the timing of meiosis II between the two replications, that is, between the two females. Second, among eggs from a single female, asynchrony was observed as different eggs entered meiosis I at different times. Third, asynchrony sometimes occurred among chromosomes of the same egg. For example, we observed that one group of dyads from meiosis I still remained as sister chromatids while the other group of dyads divided and moved toward opposite poles. In contrast, dyads separate synchronously during meiosis II in diploid (Guo *et al.*, 1992) and triploid (Guo and Allen, 1994b, and this study) Pacific oyster eggs.

The mechanism behind the formation of tetraploid embryos was indicated by the chromosome segregations observed in this study. United bipolar segregation was confirmed and would undoubtedly lead to the formation of tetraploids, supporting the hypothesis that tetraploids were produced in this way (Guo, 1991; Guo and Allen, 1994b). Tetraploids could also be produced by certain separated bipolar segregations. The two sets of dividing chromosomes need not have equal numbers of chromosomes so long as the anaphase/telophase II was oriented perpendicular to the egg membrane. In this case, the equational division of the dyads from both groups would yield 30 chromosomes at the periphery of the egg and 30

in the interior. Finally, the two peripheral chromosome sets would have to be released as PB2. When combined with 10 paternal chromosomes, the zygote would then contain a total of 40. Because the frequency of separated bipolar segregation was low (2.5%), it is reasonable to suppose that separated bipolar segregation accounts for few, if any, tetraploids. The other segregation patterns (tripolar segregation, incomplete united bipolar segregation, and unclassified patterns) would give rise to aneuploids with varying chromosome compositions, but rarely tetraploids. Overall, we conclude that united bipolar segregation is the major cause for the formation of tetraploids. Further support for this conclusion could be obtained by observing the correspondence between the frequency of united bipolar segregations and the percentage of tetraploidy across a number of replicates. These data are unavailable in this study.

If united bipolar segregation is the principal mechanism for tetraploid induction in Pacific oyster eggs, there is an important practical implication. Tetraploids are an important new tool in shellfish aquaculture because they can be crossed to diploids to yield populations that are 100% triploid (Guo *et al.*, 1996). In turn, triploid progeny are a valuable product for growers because of the value-added benefit of their reproductive sterility (Allen, 1988). Therefore, the production of tetraploid broodstock for use in hatcheries will be increasingly important. This study indicates that the high mortality among larvae from spawns to make tetraploids (*i.e.*, $3n \times 2n$ crosses) (Guo and Allen, 1994b, and unpubl. data) is a normal and inescapable consequence of this technique for making tetraploids—the cost of doing business. Only about 12% of all segregations in PB1-inhibited triploid eggs can be expected to yield tetraploid embryos and larvae. The rest would consist of a few euploids and various aneuploids, some of which will be viable (Guo and Allen, 1994a, b). Of course, only euploids and less severe aneuploids will survive through metamorphosis. Overall, the percentage of embryos that receive viable chromosome complements following inhibition of PB1 in triploid eggs is small. Of particular interest is that the frequency of united bipolar segregations observed here (mean: 12%) is almost double the frequency observed in PB1-inhibited diploid eggs (Guo *et al.*, 1992).

The patterns of chromosome segregation in eggs from triploids, following inhibition of PB1, were similar to those in diploid eggs following the same treatment, which implies that similar mechanisms underlie the segregation of chromosomes during meiosis II. We speculate that the behavior of centrioles is ultimately responsible for these patterns of chromosome segregation. Centrioles are intimately involved in the formation of meiotic spindles in *Mytilus edulis* (Longo and Anderson, 1969) and *Spisula solidissima* (Longo and Anderson, 1970). The role of centrioles in establishing multipolar meiotic apparatuses was also suggested in a recent investigation concerning the effect of cytochalasin B during meiotic maturation of diploid eggs from *Crassostrea gigas* (Longo *et al.*, 1993). In that study, inhibition of PB1 caused the formation of a tripolar spindle. The authors suggested that the centrioles normally extruded with the first polar body would participate in the organization of one pole of the tripolar spindle. The other centriole, normally remaining in the egg, divided and was responsible for the organization of two poles of the tripolar spindle. It is likely that the same mechanism underlies the formation of tripolar configurations observed in this study. Furthermore, the united bipolar segregation could be the consequence of either the dysfunction of the peripheral centrioles or the nondivision of inner centrioles; separated bipolar segregation could arise from the division of both the resident and peripheral centrioles. However, these hypothetical mechanisms by which chromosomes segregate during meiosis II following inhibition of PB1 remain to be proved in future studies.

## Acknowledgments

We are grateful to Mr. Geoff Denham for general lab assistance. This project was partly sponsored by the NOAA U.S.—China Marine and Fisheries Science and Technology Agreement in Living Marine Resources and partly by NOAA Sea Grant B/T-4. This is NJAES Publication Number D-32100-2-97.

## Literature Cited

**Allen, S. K., Jr. 1986.** Genetic manipulations: critical review of methods and performances in shellfish. Pp. 127–143 in *Selection, Hybridization and Genetic Engineering in Aquaculture*, Vol. II, K. Tiews, ed. Heenemann Verlag, Berlin.

**Allen, S. K., Jr. 1988.** Triploid oysters ensure year-round supply. *Oceanus* **31:** 58–63.

**Allen, S. K., Jr., S. L. Downing, and K. K. Chew. 1989.** *Hatchery Manual for Producing Triploid Oysters.* University of Washington Press, Seattle, WA.

**Darlington, C. D. 1965.** *Cytology.* 3rd Ed. J. & A. Churchill, London.

**Gosden, C. M., M. O. Wright, W. G. Paterson, and K. A. Grant. 1976.** Clinical details, cytogenetic studies and cellular physiology of a 69, XXX fetus with comments on the biological effect of triploidy in man. *J. Med Genet.* **13:** 371–380.

**Guo, X. 1991.** Studies on tetraploid induction in the Pacific oysters, *Crassostrea gigas* (Thunberg). Ph. D. Dissertation, University of Washington, Seattle, WA.

**Guo, X., and S. K. Allen, Jr. 1994a.** Reproductive potential and genetics of triploid Pacific oysters, Crassostrea gigas (Thunberg). *Biol. Bull.* **187:** 309–318.

**Guo, X., and S. K. Allen, Jr. 1994b.** Viable tetraploids in the Pacific oyster (*Crassostrea gigas* Thunberg) produced by inhibiting polar body I in eggs from triploids. *Mol. Mar. Biol. Biotechnol.* **3:** 42–50.

**Guo, X., G. A. DeBrosse, and S. K. Allen, Jr. 1996.** All triploid Pacific oysters (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. *Aquaculture* **142:** 149–161.

**Guo, X., W. K. Hershberger, K. Cooper, and K. K. Chew. 1992.** Genetic consequences of blocking polar body I with cytochalasin B in fertilized eggs of the Pacific oyster, *Crassostrea gigas* II. Segregation of chromosomes. *Biol. Bull.* **183:** 387–393.

**Komaru, A., and K. I. Wada. 1994.** Meiotic maturation and progeny of oocytes from triploid Japanese pearl oysters (*Pinctada fucata martensii*) fertilized with spermatozoa from diploids. *Aquaculture* **120:** 61–70.

**Loidl, J., and G. H. Jones. 1986.** Synaptonemal complex spreading in *Allium*. I. Triploid *A. sphaerocephalon*. *Chromosoma* **93:** 420–428.

**Longo, F. J., and E. Anderson. 1969.** Cytological aspects of fertilization in the Lamellibranch, *Mytilus edulis*. I. Polar body formation and development of the female pronucleaus. *J. Exp. Zool.* **172:** 69–96.

**Longo, F. J., and E. Anderson. 1970.** An ultrastructural analysis of fertilization in the surf clam, *Spisula solidissima* I. Polar body formation and development of the female pronucleus. *J. Ultrastruct. Res.* **33:** 495–514.

**Longo, F. J., L. Mathews, and D. Hedgecock. 1993.** Morphogenesis of maternal and paternal genomes in fertilized oyster eggs (*Crassostrea gigas*): effects of cytochalasin B at different periods during meiotic maturation. *Biol. Bull.* **185:** 197–214.

**Luciani, J. M., M. Devictor, J. Bour, M. Freund, and A. Stahl. 1978.** The meiotic behavior of triploidy in a human 69, XXX fetus. *Cytogenet. Cell Genet.* **20:** 226–231.